IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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For: **Apoptosis Inducing Molecule I**	

Declaration of Ann Ferrie
Ruben Exhibit #158

Paper No. EEF

Filed on Behalf of Party Ruben:

By: Samuel B. Abrams, Esq.
Margaret B. Brivanlou, Esq.
JONES DAY
222 East 41st Street
New York, New York 10017
Tel: (212) 326-3939
Fax: (212) 755-7306

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES
(Administrative Patent Judge Sally Gardner Lane)

STEVEN M. RUBEN

Junior Party,
(Application No. 08/816,981),

v.

STEVEN R. WILEY
and RAYMOND G. GOODWIN

Senior Party,
(Patent No. 5,763,223).

Patent Interference No. 105,077

DECLARATION OF ANN FERRIE

Ruben EXHIBIT 2158
Ruben v. Wiley et al.
Interference 105,077

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Filed on Behalf of Party Ruben:

By: Samuel B. Abrams, Esq.
Margaret B. Brivanlou, Esq.
JONES DAY
222 East 41st Street
New York, New York 10017
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Wiley Exhibit 1050
Ruben v. Wiley
Interference 105,077

MAP

Ruben EXHIBIT 2086
Ruben v. Wiley et al.
Interference No. 105,077
RX 2086

Paper No. _____

Filed on Behalf of Party Ruben:

By: Samuel B. Abrams, Esq.
Margaret B. Brivanlou, Esq.
JONES DAY
222 East 41st Street
New York, New York 10017
Tel: (212) 326-3939
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DECLARATION OF ANN FERRIE

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I, ANN FERRIE, declare and state as follows:

1. I joined Human Genome Sciences, Inc. (HGS) in March 1993 as a Research Associate under the direct supervision of Steven M. Ruben, who at that time was a Scientist in the Department of Molecular Biology. I worked as a Research Associate under Dr. Ruben's supervision until I left HGS in June 1998. I have been asked by patent counsel for HGS to describe my role and the experiments I carried out at HGS in connection with the development of the protein known as Apoptosis Inducing Molecule I ("AIM-I") from the inception of the project through March 14, 1996. From the initiation of my work on AIM-I in 1993, through March 14, 1996, AIM-I was continuously a priority project of mine. At that time, I was known as Ann M. Kim. During this time period, I maintained numbered laboratory notebooks in which I recorded my daily activities, including benchwork experiments, meetings, and absences from the laboratory. I refer to the following notebooks of mine in this declaration:

HGS Notebook 82 - Ann Kim #3 - RE87

HGS Notebook 127 - Ann Kim #5 - RE88

HGS Notebook 168 - Ann Kim #6 - RE89

HGS Notebook 202 - Ann Kim #7 - RE90

HGS Notebook 236 - Ann Kim #8 - RE91

HGS Notebook 270 - Ann Kim #9 - RE92

HGS Notebook 345 - Ann Kim #10 - RE93

HGS Notebook 405 - Ann Kim #11 - RE94

HGS Notebook 489 - Ann Kim #12 - RE95

CLONING AND SEQUENCING HTPAN08

2. In 1993, Dr. Ruben selected clone HTPAN08 from the HGS EST database for further investigation. Dr. Ruben communicated to me at that time that his interest in this clone was based on its homology to Fas Ligand (FasL) and Tumor Necrosis Factor α (TNFα). Dr. Ruben instructed me, as his research assistant, to place orders for oligonucleotides that served as the basis for nucleotide sequencing of the HTPAN08 clone. The first objective of the sequencing was to determine whether HTPAN08 indeed encoded a full-length TNF family protein and to identify sequences useful in expressing AIM-I to confirm that AIM-I has the expected FasL/TNFα-apoptotic activity.

3. As sequencing was periodically carried out, Dr. Ruben instructed me to perform BLAST analyses of the resultant nucleotide and encoded amino acid sequence against public databases. Initially, Dr. Ruben directly instructed me on how to carry out and analyze the BLAST analyses. Eventually, I was able to perform the BLASTs myself. Whether or not Dr. Ruben directly assisted me in carrying out the BLAST, I would meet with Dr. Ruben either the same day or within a week to discuss any new sequencing and BLAST results. In general, I met with Dr. Ruben to discuss all my experimental results on a regular basis, generally two to three times per week during the 1993-1994 time frame. After this time frame, as I became more experienced, Dr. Ruben continued to supervise and direct my work, but I became more independent in carrying out my work.

4. In addition to laboratory notebook records prepared by each individual bench researcher, HGS maintains a separate computerized database known during the 1994-1996 time frame as "IRIS". The IRIS database summarizes company projects on a Project Worksheet form

that is an integral part of the technical information record-keeping process at HGS. The content of a Project Worksheet form entered and maintained in the IRIS database is updated by the responsible HGS staff scientists as he or she deems necessary. The entire content of the electronic database during the time I worked on AIM-I at HGS was periodically recorded on tape and archived.

5. In addition to my numbered laboratory notebooks in which I recorded my daily activities (*e.g.*, RE87-95), I maintained a loose leaf three ringed binder of sequencing orders and analyses specifically for AIM-I (HTPAN08). A copy of the contents of this binder is RE96 Each sequencing run was given a designated code number based on the clone identifier (clone i.d.) HTPAN08 and the oligonucleotide primer that was used to carry out the sequencing reaction. These sequence order records in RE96 include dated printouts from an Applied Biosystems automated oligonucleotide synthesizer that indicates the date of the oligonucleotide synthesis run ("run date"), as well as the sequence and code designation of the particular primer. The sequence records of RE96 also include an HGS sequencing results summary form filled out and dated by hand by the sequencing technician who carried out the sequencing run, which detailed the raw data from the sequencing run. On these sequencing results summary forms, the technician would enter the date on which the results were entered into an electronic results folder which I accessed. In addition, RE96 contains dated printouts of sequences analyses, including BLAST searches and sequence alignments, that I performed at Dr. Ruben's direction.

6. On September 7, 1993, in the early stages of this sequencing process, I inoculated two 5 ml TB cultures with colonies containing HTPAN08 DNA, a cDNA encoding a portion of

the protein later referred to as apoptosis inducing molecule-I, or AIM-I RE87 page 43).

7. On September 20, 1993, I began making midi preps of clone HTPAN08 ("Tumor Necrosis Factor α") and began preparing oligonucleotide primers ("F" for forward, "R" for reverse) for sequencing the clone (RE87 , pages 72, 77, 78, 84, 143).

8. Initial sequencing indicated that the original HTPAN08 clone encoded a partial-length protein. It was eventually determined that this partial cDNA clone encoded 147 carboxy terminal amino acid residues of AIM-I (*i.e.* the region of TNF ligand family members having the greatest homology) but was missing the 5' end encoding 134 amino terminal residues of AIM-I.

9. I have been shown IRIS Project Worksheets for clone HTPAN08 which have been represented to me as printouts from an archive tape from the HGS electronic database dated April 29, 1994 RE97 and RE98). On February 8, 1994, I opened up a new project for HTPAN08 pursuant to Dr. Ruben's instructions (RE98). On February 9, 1994, I had obtained new sequence information of HTPAN08 (RE96 , page 24) and had BLASTed this new HTPAN08 sequence against a database of publicly available proteins (RE98). The BLAST results of the HTPAN08 encoded protein showed a high degree of homology with rat FasL, TNFα, and lymphotoxin beta, members of the TNF ligand family. I communicated these BLAST results to Dr. Ruben on or about February 9, 1994. By no later than February 9, 1994, Dr. Ruben had communicated to me his interest in pursuing the protein encoded by HTPAN08 as an apoptosis inducing agent for developing therapeutics, *e.g.*, to treat cancer, and instructed me to screen the HTP cDNA library from which the original HTPAN08 clone was isolated for a full length cDNA clone. This screening process began on February 3, 1994 (RE88 , page 7). By no later than April 29, 1994, I had selected this information from the BLAST results of February

9, 1994 as relevant to our analysis of HTPAN08 and placed it into the project records, under Dr. Ruben's direction. Thus, on or about February 9, 1994 and no later than April 29, 1994, Dr. Ruben had communicated to me that clone HTPAN08 (AIM-I) encoded a protein with a high degree of homology to FasL and TNFα, and that the protein encoded by HTPAN08 (AIM-I) would have apoptotic activity.

10. By March 3, 1994, I had isolated fourteen screened clones from the HTP cDNA library which were designated consecutively HTPAN08SO1 through HTPAN08SO14. RE88 page 67. I pursued clones SO3, SO4, SO13, and SO14 further to determine if any of them contained a full length clone (RE88 , page 67). On May 6, 1994, it was determined that clones SO4, SO13, and SO14 were full-length (RE88 , page 132).

11. On May 5, 1994, Dr. Ruben directly instructed me in aligning HTPAN08-encoded amino acid sequence with the amino acid sequence of rat FasL (public database accession number U03470) using TFASTA (GCG package) (I RE96 , page 38). Thus, by May 5, 1994, Dr. Ruben had communicated to me that clone HTPAN08 (AIM-I) encoded a protein with a high degree of homology to FasL, and that based on the identification of such a full-length clone with such homology, that the protein encoded by HTPAN08 (AIM-I) would have apoptotic activity and therefore would have utility in treating cancer.

12. By June 20, 1994, I had determined and communicated to Dr. Ruben that the screened clone HTPAN08SO4 contained a full-length cDNA encoding a complete AIM-I protein (see IRIS Sequence Worksheet for HTPAN08XX - RE100 . On June 20, 1994, under Dr. Ruben's instruction, I entered a full-length AIM-I sequence into the IRIS database RE100) which I later discovered contained minor sequencing errors. This sequence was determined by

sequencing the HTPAN08SO4 clone and was designated HTPAN08XX. Thus, by June 20, 1994, Dr. Ruben had communicated to me that clone HTPAN08SO4 (AIM-I) encoded a full-length protein with a high degree of homology to FasL and TNFα, and that based on the identification of such a full-length clone with such homology, that the protein encoded by HTPAN08SO4 (AIM-I) would have apoptotic activity.

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13. On August ~~8~~, 1994, under Dr. Ruben's direction, I aligned the AIM-I protein amino acid sequence with several other members of the TNF ligand family to highlight the regions of homology among the proteins, including TNF alpha, TNF beta, Fas ligand (FasL), and HUVEO91, a TNF ligand family member that Dr. Guo-Liang Yu, then a scientist at HGS, was studying (RE96 , pages ~~79-80~~ 90-93 AMF 08/03/04). I also printed out a translation of a full-length nucleotide sequence for HTPAN08XX on August 8, 1994 (RE96 , pages 73-78).

14. On August 9, 1994, under Dr. Ruben's instruction, I performed a BLAST analysis of the HTPAN08XX amino acid sequence which revealed high degrees of similarity between AIM-I and several species of TNF alpha and Fas ligand proteins (RE96 , pages ~~71-72~~ 83-85 AMF 08/03/04).

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15. I discussed the August ~~8~~, 1994 alignment and the August 9, 1994 BLAST result with Dr. Ruben within a week of August 9, 1994, and it confirmed that the AIM-I protein encoded by HTPAN08SO4 was a full-length member of the TNF ligand family with a high degree of homology to TNFα and FasL and, like those two proteins, would be useful in inducing apoptosis in certain cells, as Dr. Ruben expressed in the February 2, 1994 project report RE97 page 1).

AIM-I Sequence Refinement

16. After the HTPAN08XX sequence was entered into IRIS, I noticed that it contained a few minor sequence errors. I detected these sequencing errors through careful but routine sequencing that I had directly carried out in the laboratory of various portions of the HTPAN08SO4 clone. Once I had communicated the correct nucleotide sequence to Dr. Ruben, Dr. Ruben instructed me to enter the correct sequence into IRIS. Thus, I entered a refined AIM-I sequence with these minor sequencing errors corrected. On August 8, 1994, I printed out the corrected nucleotide sequence and corresponding amino acid sequence of AIM-I (RE96 at pages 73-78), which was referred to as HTPAN08XX instead of HTPAN08xy, as the corrected sequence became known. I also entered the corrected sequence of AIM-I, with the designation HTPAN08xy, into the IRIS database on November 15, 1994 (IRIS Sequence Worksheet for HTPAN08xy - see RE 101 . I generated a printout of the protein encoded by the HTPAN08xy sequence that was generated on January 20, 1995 (I RE96 , pages 115-116), and a printout of the 846-nucleotide sequence of the HTPAN08xy open reading frame on February 21, 1995 RE96 page 120-122).

PRODUCTION AND CHARACTERIZATION OF AIM-I PROTEIN

17. In addition to sequencing AIM-I clones (*e.g.*, HTPAN08 and HTPAN08SO4), I worked under Dr. Ruben's supervision on developing reagents and characterizing this gene so that AIM-I could be pursued further in HGS's interdepartmental program as a therapeutic candidate. The primary focus of my role in this development was to make recombinant constructs of the AIM-I cDNA and to express the cDNA in recombinant host cells to produce

protein for analyzing its biological properties. I have been asked by patent counsel to HGS to describe the activities I carried out under Dr. Ruben's supervision in this regard.

18. In January 1995, I successfully expressed and purified AIM-I protein, as described in detail below. I generated a number of expression constructs, each containing one of two forms of AIM-I: (a) construct HTPAN08S04 51bp, containing an 846-base pair open reading frame, was used to generate a protein with translation beginning at the first residue of the AIM-I protein, *i.e.* methionine 1 ("Met-1) and; (b) construct HTPAN08S04 185bp, containing a 714-base pair open reading frame, was used to generate a protein with translation beginning at the 45th residue of the AIM-I protein, *i.e.* methionine 45 ("Met-45). On January 20, 1995, printouts of the protein sequences of these two forms of AIM-I, including an analysis of their codon usage, were prepared (RE96 , pages 115-116 and 113-114, respectively). On February 21, 1995, I printed out nucleotide sequences of these two forms of AIM-I, labeled "htpan08orf (1>846)" and "htpan08 185bporf (1>714)", respectively, in relation to the sequence run data (RE96 , pages 120-122 and pages 117-119, respectively).

19. I initially cloned these two forms of AIM-I into three different vectors, pD10, pQE60 and pA2. pD10 and pQE60 are bacterial expression vectors whereas pA2 is a baculovirus vector for expression in Sf9 insect cells.

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20. On ~~July 26,~~ 1994, I grew the HTPAN08S04 clone for a large scale-up plasmid preparation to provide the raw material (DNA) needed for construction of the various AIM-I expression constructs mentioned above (~~R~~ RE88 ~~page 136~~). Also, production of the AIM-I protein via *in vitro* transcription and translation required that a large amount of template DNA be produced. At this time, the HTPAN08S04 plasmid clone was referred to as "TNF alpha-Fas" in

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recognition of the homology of the protein it encoded to the TNF alpha and Fas ligand proteins (RE89 , page 136). On August 31, 1994, a large-scale restriction digest of the HTPAN08SO4 plasmid was performed to release the cDNA insert. The full-length HTPAN08SO4 clone was digested with EcoRI and XhoRI, and an aliquot of the digest was run on an agarose gel. Since the digestion appeared to be partial, it was continued overnight in order to facilitate completion (I RE90 , page 44). On September 1, 1994, an aliquot of the overnight digest was run on a gel, and digestion appeared to be complete. The DNA was therefore cleaned and resuspended in TE buffer for future use (I RE90 . page 46).

The pA2 Insect Cell Expression Construct

21. As part of the foregoing process, an effort was commenced on September 2, 1994 to construct a baculovirus expression vector (dubbed the "pA2 construct") for use in expression of the AIM-I protein in insect cells. First, PCR was performed using the HTPAN08S04 clone as a template and primers containing nucleotides encoding the Asp718 and BamHI restriction sites. The products of the PCR reaction were run on a gel, precipitated with polyethylene glycol (PEG), and resuspended in TE (RE90 , page 57).

22. On September 6, 1994, the PCR products were restriction digested and the completion of the digest was confirmed on a gel. The restriction products were cleaned and a ligation reaction was prepared with the restricted HTPAN08S04 fragment and pA2. This ligation reaction was incubated overnight (I RE90 , pages 58, 69). On September 7, 1994, the ligation reaction products were used to transform DH5α competent cells with varying concentrations of the pA2 construct. These cells were plated out for overnight growth

RE90 page 70).

23. On September 8, 1994, I picked colonies from the overnight plates and inoculated them into LB/amp medium for a four hour incubation. I then performed PCR to confirm the presence of inserts in the picked colonies, using the primers of September 2, 1994, containing the restriction sites Asp718 and BamHI (primers 7690 and 7689; see RE96 at pages 106-107), to perform the reaction. Colonies so identified as having inserts were then inoculated into TB/amp medium for overnight incubation (I RE90 , pages 70-72).

24. On September 9, 1994, I performed boiling mini-preps of the overnight TB/amp cultures, then size-screened them on a gel to ensure positive clones. The DNA was digested with BamHI and Asp718, and the digested fragments were run on a gel to confirm that they contained inserts. The clones were then submitted for sequence confirmation. Glycerol stocks of positive clones were also prepared and frozen for future use (RE90 , pages 72, 81).

25. On September 12, 1994, I inoculated positive AIM-I clones dubbed HTPAN08-B01 and -B02 into TB/amp and set them up for incubation overnight (RE90 , page 82).

26. On September 13, 1994, I performed a maxi-prep DNA isolation procedure on the -B01 and -B02 clones. I ran DNA samples on a gel and stored the remaining DNA at 4°C RE90 , pages 82-83).

27. On November 21, 1994, I made new primers for cloning AIM-I into the pA2 baculovirus expression vector. The 5' primers, No. 2792 (5' BamHI 51bp) and No. 2793 (5' BamHI 185bp), were paired with 3' primer No. 7690 (3' Asp718) in separate reactions for the production of two AIM-I inserts having alternative translation start sites (*i.e.* at base pair 51 and

185, respectively). Using these primers and AIM-I template DNA (*i.e.* HTPAN08SO4), I PCR amplified inserts for cloning into the BamHI/Asp718 sites of the pA2 expression vector. Once amplified, I cut the inserts with BamHI/Asp718 restriction enzymes to prepare the ends for cloning into the pA2 vector (RE91 , pages 56-57).

28. November 22-29, 1994 encompassed the Thanksgiving holiday.

29. On November 30, 1994, I gel purified the cut inserts for subcloning into the pA2 vector (RE91 , pages 58 and 62). I also performed ligation of the purified inserts into pA2 (RE91 , page 58).

30. On December 1-5, 1994, I transformed a portion of the ligation products into M15/Rep4 cells, plated, and maintained for colony formation (RE91 page 58).

31. On December 2-5, 1994, I transformed another portion of the ligation products into DH5α cells, plated, and maintained for colony formation (RE91 , page 67).

32. On December 6, 1994, I inoculated positive colonies into culture for PCR analysis (RE91 , page 67).

33. On December 7, 1994, I performed PCR analysis on the cultured positive colonies to check for the presence of HTPAN08 (*i.e.* AIM-I) inserts in the pA2 vector. I also inoculated positive colonies for overnight miniprep culture (RE91 , pages 67-68).

34. On December 8, 1994, I purified DNA from the overnight miniprep cultures, analyzed uncut on a gel, and digested the DNA with BamHI/Asp718 (RE91 , pages 68, 89).

35. On December 9, 1994, the BamHI/Asp718 digestion I had performed on December 8, 1994, appeared to be unsuccessful. Clones were submitted for sequencing using

FP06 and RP19 primers (RE91 , page 90).

36. On January 11, 1995, I obtained new primers for the pA2 baculovirus expression vector. The new primers included a more efficient Kozak consensus sequence for translation initiation (RE91 , pages 90, 125). I used the new primers for amplification of the AIM-I coding sequences (RE91 , page 90).

37. On January 12, 1995, I performed PCR analysis on the 51bp and 185bp AIM-I inserts in the pA2 expression vector, and DNA fragments were digested for cloning (RE91 , page 125).

38. On January 13, 1995, I performed gel purification of the restricted AIM-I inserts and ligation into the pA2 vector over the weekend, *i.e.*, until January 16, 1995 (RE91 , pages 126-7).

39. On January 16, 1995, I transformed the ligations from January 13, 1995 into M15 chemically competent cells and cultured the cells overnight, *i.e.*, until January 17, 1995 RE91 page 127).

40. On January 17, 1995, I cultured colonies obtained from the January 16, 1995 transformation (RE91 , page 128).

41. On January 18, 1995, I performed minipreps on the cultures confirm that I had obtained pA2 HTPAN08S04 51bp and 185bp clones (RE91 , pages 135-7).

42. On January 23, 1995, I prepared maxipreps of constructs pA2/HTPAN08S04 51bp and 185bp RE91 , pages 150-51).

43. On January 26, 1995, I received new primers for cloning AIM-I (*i.e.*

HTPAN08S04) into the pA2 vector (Primer Nos. 9111 and 9112), and deprotected them overnight (RE92 , page 5). With these primers, I performed PCR reactions on January 27, 1995 to generate new AIM-I inserts, and digested the PCR products with XbaI and BamHI, gel-purified them, and ligated them at 16°C over the weekend, *i.e.*, until January 30, 1995 (RE92 , pages 5-8).

44. On January 30, 1995, I transformed and plated the weekend ligations, and incubated the plates overnight (RE92 , page 8).

45. On January 31, 1995, I performed PCR on positive colonies from the overnight incubation to amplify inserts (RE92 , pages 8-10).

46. On February 1, 1995, I analyzed the amplified inserts by agarose gel electrophoresis RE92 , pages 10-11).

47. On February 15, 1995, I submitted baculovirus clones for sequencing (HTPAN08S04 51bp and 185bp in pA2); I also submitted these clones to the Protein Expression Department for recombinant baculovirus production (RE92 , page 29).

The pD10/pQE9 Bacterial Expression Construct

48. In late September 1994, I began working, under Dr. Ruben's supervision, on a bacterial expression construct for the AIM-I protein, referred to alternatively as the pD10 or pQE9 construct. Bacterial expression of AIM-I was important for providing an abundant source of AIM-I antigen used for the production of polyclonal antibodies. Moreover, AIM-I expressed in bacteria was important for providing a source of protein therapeutic agent for use in assays

designed to demonstrate apoptotic activity. The activities summarized below describe some of the work performed during the development of the pD10/pQE9 AIM-I construct.

49. On September 28, 1994, I performed PCR with HTPAN08S04 DNA as template and primers containing engineered BamHI and XbaI restriction sites. The product was run on a gel, precipitated with polyethylene glycol (PEG), and resuspended in TE buffer (RE90 , pages 111-112).

50. On September 29, 1994, I ran the PCR product on a low-melt agarose gel, excised the PCR product band from the gel, and cleaned the band using gene clean. The cleaned product was then digested with BamHI and XbaI (RE90 page 112).

51. On September 30, 1994, I ran PCR product digests from September 29, 1994 on a low-melt gel and two desired bands were visualized at 0.9 kilobase (kb) and 1.6 kb. These bands were excised, cleaned, and ligated into the pD10 vector which had been previously digested with BamHI and XbaI. *E. coli* M15 cells were then transformed with the ligation reaction products and plated for incubation at room temperature over the weekend (RE90 , pages 113-114).

52. On October 3, 1994, I picked colonies from the weekend plates and cultured them in LB/amp/kan medium at 37°C for 4 hours. I then screened these cultures by PCR for the presence of insert. I again transformed the M15 cells with the remaining portion of the ligation reaction plated the cells for overnight incubation (RE90 , pages 115-116).

53. On October 4, 1994, I picked colonies from the previous day's plates and cultured them in LB/amp/kan. These cultures were screened by PCR for the presence of insert, and only one positive culture was found. As a result, I digested fresh pD10 vector with BamHI and XbaI

was prepared and also cultured pD10 stock for maxi-prep DNA isolation was performed

RE90 pages 116, 123-124).

54. On October 5, 1994, I set up ligation reactions for overnight incubation at 16°C

(RE90 page 124).

55. On October 6, 1994, I transformed *E. coli* M15 cells with the previous day's ligation and plated the cells for overnight incubation (RE90 , pages 124-125).

56. On October 7, 1994, I noted poor colony growth on the previous day's ligation plates. Accordingly, I began to remake the pD10 vector and insert fragments RE90 , page 125).

57. On October 10, 1994, I digested the pD10 vector overnight with BamHI and XbaI (RE90 , page 125).

58. On October 11, 1994, I ran the digest on a low-melt agarose gel and excised and cleaned the DNA band corresponding to cut pD10 vector. I also set up a digest of AIM-I DNA, using the HTPAN08 PCR product of September 28, 1994, with BamHI and XbaI, and ran the products of the digest on a low-melt gel. I then excised and cleaned the desired DNA band

RE90 pages 126, 146).

59. On October 12, 1994, I set up a ligation of AIM-I DNA (*i.e.* HTPAN08) into the bacterial expression vector pD10 for overnight incubation RE90 , pages 146-147).

60. On October 13, 1994, I transformed M15 cells with the ligation products from October 12, 1994, and plated for overnight incubation RE90 page 147).

61. On October 14, 1994, the plates from October 13, 1994 showed good colony

growth, and I picked colonies for expansion in LB/amp/kan medium. These cultures were screened for the presence of insert by PCR using primers 2499 and 2500 (RE96, pages 109-110), but no insert was detected (RE90, page 148).

62. On October 19, 1994, I performed PCR to amplify AIM-I DNA (*i.e.* HTPAN08SO4) using the primers containing the engineered BamHI and XbaI restriction sites from September 28, 1994. The PCR products were run on a gel, PEG precipitated, and resuspended. I set up digests of the PCR products for overnight incubation with BamHI and XbaI (RE90, page 149-150).

63. On October 20, 1994, I ran the digestion products on a low-melt gel and excised the desired bands from the gel and cleaned them. These bands were ligated overnight into the pD10 vector (the HTPAN08/pD10 ligations) (RE90, page 150; RE91, page 3).

64. On October 21-23, 1994, I transformed M15 cells with the ligation products, plated, and incubated the transformed cells at room temperature over the weekend (RE91, page 3).

65. On Monday, October 24, 1994, I noted that the weekend plates grew well, and picked colonies for culture in LB/amp/kan medium. I screened these cultures by PCR for the presence of insert, but did not observe any positives. At this point, I determined that it would likely be necessary to retry the cloning procedure using new primers (RE91, pages 4-5).

66. On October 26, 1994, I again performed PCR of AIM-I DNA using HTPAN08SO4 DNA as template and BamHI/XbaI primers. The PCR products were run on a gel, PEG precipitated, cleaned, and incubated overnight with BamHI/XbaI restriction enzymes

(RE91 , pages 5-7).

67. On October 27, 1994, I ran the overnight PCR product digests on a low melting point gel. The desired band containing the AIM-I DNA was excised, cleaned, and ligated overnight into the pD10 bacterial expression vector (RE91 , pages 7-9).

68. On October 28-30, 1994, I transformed *E. coli* M15 cells with the overnight ligation products, plated the transformed cells, and incubated them over the weekend (RE91 , pages 9-10).

69. On October 31, 1994, I did not remove the weekend plates from the incubator, but instead allowed them to continue growing. I performed PCR on several of the colonies to determine whether any insert was cloned into the vector, but did not identify positive colonies. I next performed PCR of fresh AIM-I DNA using the HTPAN08SO4 DNA as template and both old and new BamHI/XbaI primers (RE91 , pages 10 and 13).

70. On November 1, 1994, I PEG precipitated, cleaned, and digested overnight the PCR-amplified fresh AIM-I DNA with BamHI/XbaI restriction enzymes RE91 page 14).

71. On November 2, 1994, I ran the digested PCR-amplified AIM-I DNA on a low melting point gel, excised, cleaned, and ligated overnight the digested DNA into the pD10 vector (RE91 , pages 14-16).

72. On November 3, 1994, I transformed *E. coli* M15 cells with the ligation products, plated the cells, and incubated them overnight (RE91 , pages 16-17).

73. On November 4, 1994, colonies were picked from the overnight plates, cultured them in LB/amp/kan medium, and screened the cultures by PCR for the presence of inserts

RE91 , page 17).

74. On November 7, 1994, I performed gel analysis of the PCR screen for inserts revealed that no colonies were positive for the presence of the AIM-1 insert. Accordingly, I placed a request for production of new primers in preparation for another attempt at subcloning AIM-1 into the pD10 bacterial expression vector (RE91 , page 18).

75. On November 10, 1994, I received a new 5' primer (RE91 , page 18 and RE96 page 112). On November 11, 1994, I received a new 3' primer (RE91 , page 18). On November 12, 1994, I performed PCR amplification of AIM-1 DNA from the HTPAN08 template using these new BamHI/XbaI primers (RE91 , page 18). The PCR products were run on a gel, PEG precipitated, cleaned, and digested over the weekend with BamHI/XbaI (RE91 page 39).

76. On November 14, 1994, I ran the digests on a low melting point gel and excised, cleaned, and ligated the desired band into the pD10 vector. I transformed the ligation products into *E. coli* M15 cells, and then plated the cells for overnight incubation (RE91 , pages 39-41).

77. On November 15, 1994, I picked colonies from the overnight plates, cultured the colonies in LB/amp/kan medium, and screened the cultures by PCR for the presence of inserts. Many positives were detected, indicating that a successful subcloning into the pD10 bacterial expression vector had finally been achieved and suggesting that earlier success had indeed been precluded by defective BamHI/XbaI primers. I transformed more *E. coli* M15 cells with the remainder of the successful ligation reaction and plated the transformations for overnight incubation (RE91 , pages 41-42 and 47).

78. On November 16, 1994, I prepared boiling minipreps of positive cultures. I ran the miniprep DNA on a gel, digested it with BAMHI/XbaI, and again ran it on a gel. The gel results indicated a successful digest, so I submitted samples of the DNA for sequencing to confirm the correctness of the finished AIM-I/pD10 bacterial expression construct (*i.e.* HTPAN08/pD10). I also performed a back-up ligation which I transformed into M15 cells, and plated the cells for overnight incubation (RE91 , pages 48 and 53).

79. On November 18, 1994, I performed a midi-prep of HTPAN08/pD10 and quantitated the resulting DNA (RE91 pages 54-55).

80. On January 11, 1995, new primers were made for the pD10 bacterial expression vector. ~~The new primers included a more efficient Kozak consensus sequence for translation initiation~~ (RE91 , pages 90 and 125).

81. On January 13, 1995, I performed gel purification of AIM-I inserts and ligation into the pD10 vector over the weekend, *i.e.*, until January 16, 1995 (RE91 , pages 126-7).

82. On January 16, 1995, I transformed the ligations from January 13, 1995 into M15 chemically competent cells and cultured the cells overnight, *i.e.*, until January 17, 1995 RE91 ; page 127).

83. On January 17, 1995, I cultured colonies obtained from the January 16, 1995 transformation RE91 , page 128).

84. On January 18, 1995, minipreps on the cultures confirm that I had obtained pD10 HTPAN08S04 51bp and 185bp clones (RE91 , pages 135-7).

85. On January 18, 1995, minipreps were repeated to further confirm all pD10

HTPAN08S04 51bp and 185bp clones (RE91 , pages 135-7).

86. On January 27-29, 1995, I received new primers for cloning AIM-I (*i.e.* HTPAN08S04) into the pD10 vector (Primer Nos. 9113 and 9114). With these primers, I performed PCR reactions were performed on HTPAN08SO4 template to generate new expression construct inserts. I digested the PCR products with XbaI and BamHI and gel-purified and ligated the insert into pD10 at 16°C over the weekend (RE92 , pages 5-8).

87. On January 30, 1995, I transformed, plated, and cultured overnight the weekend ligations (RE92 , page 8).

88. On January 31, 1995, I performed PCR on positive colonies from the overnight cultures to amplify inserts (RE92 , pages 8-9).

89. On February 1, 1995, I analyzed the amplified inserts by agarose gel electrophoresis (RE92 , pages 10-11).

The AIM-I pQE60 Construct

90. In mid-December 1994, I began working on another AIM-I bacterial expression construct referred to as pQE60. This construct places a tag on the expressed protein to facilitate its purification.

91. On December 19, 1994, I cut the pQE60 bacterial expression vector with BamHI and NcoI, and gel purified and gene-cleaned the vector in preparation for subcloning AIM-I (*i.e.* HTPAN08S04) into it (RE91 , page 97).

92. On December 20, 1994, I digested AIM-I DNA (*i.e.* HTPAN08S04) with

NcoI/BglII for subcloning into the pQE60 vector I had prepared the previous day (RE91 pages 99-100).

93. On December 22, 1994, I gel purified the NcoI/BglII digested AIM-I insert DNA and ligated the insert into the BamHI/NcoI digested pQE60 vector (RE91 , pages 100-01).

94. On December 23, 1994, I performed PCR to generate an AIM-I insert having the methionine 45 as the start codon (*i.e.* HTPAN08S04 185bp insert) and NcoI/BglII sites for subcloning into the BamHI/NcoI-digested pQE60 vector (RE91 , page 103).

95. December 24, 1994 through January 2, 1995 encompassed the year-end holiday season.

96. On January 3, 1995, I transformed the December 22, 1994 ligation products into M15/Rep4 *E. coli* cells (RE91 , pages 101-2). The "185bp" PCR product of December 23, 1994 was also digested (RE91 page 104).

97. On January 4, 1995, I repeated the previous day's transformation because I detected contamination of the competent cells (RE91 page 102). I also started preparing a maxiprep of HTPAN08S04/pBluescript plasmid (RE91 , pages 111-112).

98. On January 5-8, 1995, I performed ligations into pQE60 using inserts HTPAN08S04 185bp from January 3, 1995 and HTPAN08S04 51bp from December 22, 1994 (RE91 , page 108). Also, I completed the maxiprep I initiated on January 4, 1995 (RE91 page 112).

99. On January 6, 1995, I quantified the DNA produced in the maxiprep of January 4, 1995 RE91 page 112).

100. On January 9, 1995, I transformed the ligations of January 5, 1995 into M15/Rep4 *E. coli* cells, and picked colonies for miniprep and PCR analyses (RE91 , page 115).

101. On January 10, 1995, I performed PCR analysis on the previous day's cultures using primers 2888/2865 for the 51 bp constructs and 2967/2865 for the 185bp constructs (RE91 , page 116).

102. On January 11, 1995, I performed agarose gel analysis on the pQE60 constructs resulting from the December 22, 1994 and January 5, 1995 ligations. Also, I inoculated more cultures with positive colonies for miniprep analysis (RE91 , pages 116, 119-20).

103. On January 12, 1995, I prepared minipreps and analyzed the uncut DNA by agarose gel electrophoresis. Digestions were set up for the pQE60 constructs from the December 22, 1994 and January 5, 1995 ligations (RE91 , pages 120-21).

104. On January 13, 1995, I performed an agarose gel analysis of the digested DNAs. Also, I submitted a single positive clone for sequencing (RE91 , page 122). Finally, I set up ligation reactions for over-the-weekend incubation (RE91 , page 127); *i.e.*, through January 16, 1995.

105. On January 16-17, 1995, I transformed into bacteria, plated, and cultured the ligations (RE91 , page 127). Additionally, I performed a repeat of the January 10, 1995 pQE60 PCR analyses using new reactions and primers (RE91 , pages 128-34).

106. On January 18, 1995, I also repeated minipreps to further confirm all pQE60 HTPAN08S04 51bp and 185bp clones (RE91 , pages 135-7).

107. On January 19, 1995, I analyzed competent cells by retransformation of prior

HTPAN08 ligations. Also, I performed PCR analysis of transformed cells and additional PCR was done to generate templates for cRNA transcription for use in further TNT analysis (RE91 , pages 138-39).

Reticulocyte Lysate Expression Of AIM-I

108. Rabbit reticulocyte lysates are commonly used for *in vitro* translation of messenger RNA transcripts. Many commercial kits are available for the performance of this assay, wherein the user provides the mRNA encoding the protein of interest. This experimental system can be used to answer a number of important questions about a protein and its encoding nucleic acids. For example, it is convenient for determining whether a correct cDNA construct has been assembled and isolated. If complementary RNA (cRNA) transcribed from a cDNA construct under study can be translated *in vitro* to produce a protein in reticulocyte lysates, then the user is assured that the construction encodes viable translation initiation and termination signals, *i.e.*, it confirms that the gene is expressible.

109. On January 18, 1995, I performed a rabbit reticulocyte lysate *in vitro* translation using the TNT system (Promega) to express the pQE60 constructs carrying AIM-I inserts (RE91 , pages 135, 141).

110. On January 20-24, 1995, I used the TNT reticulocyte lysate expression system for *in vitro* translation of AIM-I (RE91 , pages 142, 149, 152).

111. On January 24, 1995, I performed TNT analysis using the pQE60/ HTPAN08S04 185bp expression construct (RE92 , page 2).

112. On February 1, 1995, I inoculated several mini-IPTG-induction cultures for overnight growth in preparation for further ~~TNT~~ AMF 08/03/04 analysis (RE92, pages 10-12).

113. On February 2, 1995, I performed ~~TNT~~ AMF 08/03/04 inductions of protein expression and PAGE gel analysis on pD10/HTPAN08S04 51bp and 185bp bacterial expression clones (RE92, pages 12-14).

114. On February 6-10, 1995, I was on vacation (RE92 page 3).

115. By December 15, 1995, I had made additional AIM-I expression constructs, including pQE6 expression constructs (see paragraphs 191-195). On December 20, 1995, I assayed these constructs in the TNT expression reactions to determine that AIM-I was capable of being properly transcribed and translated. Thus, on December 20, 1995, protein gel analyses were performed on inserts from the AIM-I/pQE6 bacterial expression constructs described above (RE94, pages 125-26).

116. On January 26, 1996, AIM-I/pQE6 construct I again expressed in the TNT expression system (RE94 page 146).

117. As detailed below, from December 21, 1995 through January 12, 1996, I was not working in the laboratory on account of a series of holidays, vacation days, personal days, and weather closings at HGS.

Bacterial Expression Of Aim-I Protein

118. On February 13-14, 1995, I made glycerol stocks of HTPAN08S04/pD10 clone #12 (RE92, page 16).

119. On February 15, 1995, I successfully performed a large-scale IPTG induction of AIM-I (Met-45) protein expression. AIM-I was expressed using HTPAN08/pD10 185bp clone #12 and detected by PAGE gel analysis of the IPTG induction product RE92, pages 29-30). Also on February 15, 1995, I successfully purified AIM-I (Met-45) over an $NiSO_4$ column RE92, pages 29-30). I successfully expressed and purified AIM-I (Met-1) protein soon thereafter (see below at March 10, 1995).

120. On February 16, 1995, I submitted a pH 5 fraction of the large-scale IPTG preparation to the Protein Expression Department for renaturation experiments (RE92, page 30).

121. On February 21, 1995, I transformed ligations in particular (the HTPAN08/pD10 ligation of January 13, 1995 and the HTPAN08/pQE60 ligation of January 5, 1995; both were the 51bp or Met-1 construction) into *E. coli* M15/Rep4 cells (RE92 page 32).

122. On February 22, 1995, I picked and cultured 48 colonies from the pD10 and pQE60 transformations of February 21, 1995 for mini-preps and PCR analyses (RE92, page 43).

123. On February 23, 1995, I performed PCR analysis on the pD10 and pQE60 transformations of February 21, 1995 (RE92, pages 43-45).

124. On February 24, 1995, I performed mini IPTG inductions on the PCR-positive clones obtained from the February 21, 1995 transformation RE92, pages 46-47).

125. On February 28, 1995, I re-ran 16 independent bacterial expression clones on PAGE to verify expression by mini-IPTG-induction (HTPANOS04/pQE60 and /pD10 51bp

subclones). Also, I inoculated a culture for maxi-prep and performed a large-scale IPTG induction on clone #C7 (RE92 , pages 48-49).

126. On March 1, 1995, I performed PAGE gel analysis on the IPTG inductions from February 28, 1995 (RE92 page 50). I performed a maxi-prep on the culture inoculated February 28, 1995 (RE92 , page 61).

127. On March 8, 1995, I initiated a culture of HTPAN08/pD10 51bp for large-scale IPTG induction of the AIM-I protein (RE92 , page 60).

128. On March 9, 1995, I induced expression of AIM-I protein in the culture initiated on March 8, 1995 (RE92 , page 62). Four hours following the induction, I spun down the cells and froze them overnight, *i.e.*, until March 10, 1995 (RE92 , page 62).

129. March 10, 1995, I performed PAGE analysis on the previous day's induction/expression experiment, and the results indicated that AIM-I protein (Met-1) was successfully produced in bacteria (RE92 page 62). Also on March 10, 1995, I successfully purified AIM-I protein (Met-1) over an $NiSO_4$ column (RE92 , pages 62 and 69).

130. On March 14, 1995, I ran a preparative gel to purify additional AIM-I protein for antibody production (RE92 , page 71). On March 15, 1995, isolated AIM-I protein was isolated from a preparative gel slice. This AIM-I sample was produced from an HTPAN08S04 51bp construct by induction of bacterial expression on March 10, 1995 (*see* RE92 , page 72, dated March 15, 1995).

131. On May 8, 1995, I inoculated a maxiprep to grow up HTPAN08S04/pD10 51bp, a bacterial expression construct containing the full-length AIM-I insert (RE92 , page 133).

132. On May 9, 1995, I performed a large-scale induction of protein expression on the HTPAN08S04/pD10 51bp clone inoculated May 8, 1995 (RE92 , pages 133-134).

133. On May 10-11, 1995, I column-purified fractions of AIM-I protein from the large-scale induction of May 9, 1995 and analyzed the fractions by PAGE (RE92 , pages 135-136).

134. On May 12, 1995, I performed additional protein induction on clone HTPAN08S04/pD10 51bp (RE92 , page 138).

135. On May 15, 1995, I collected additional column fractions from the May 9, 1995 large scale induction at pH 8, 6, 5 and 2 (RE92 , page 139).

136. On May 17, 1995, I performed a PAGE gel analysis of the May 15, 1995 fractions (RE92 , page 140).

137. On May 18, 1995, I ran the pH 5 fraction of AIM-I on a fresh column. I collected the protein and transferred it to the Protein Expression Department for preparation on a renaturation column (RE92 page 141).

138. On May 23, 1995, I received back the renaturation column from the Protein Expression Department. I eluted renatured AIM-I protein with imidazole elution buffer and performed PAGE analysis of eluted protein was preformed (RE92 , page 141).

The AIM-I/pcDNA Mammalian Constructs

139. On July 3, 1995, I received PCR primers (primers 12305 and 12308) which were ordered for the purpose of inserting a hemagluttinin (HA) tag on the 5' and, alternatively, the 3'

end of the AIM-I protein, into each of two HTPAN08/pcDNA mammalian expression vectors for AIM-I. The pcDNA expression vectors contain a CMV promoter that provides high levels of expression in mammalian host cells. On the same day, I performed PCR reactions using the primers to generate the required fragments for making the expression constructs (RE93 pages 37-39).

140. On July 5, 1995, I set up a restriction digest of the PCR fragments and incubated overnight (RE93 , page 41).

141. On July 6, 1995, I ran the restriction digest products on a low melting point (LMP) agarose gel, and the 3' HA tagged and 5' HA tagged fragments were isolated. The pcDNA mammalian expression vector was also digested overnight with the appropriate enzymes for the two constructs on July 6, 1995 (RE93 , pages 42-44).

142. On July 10, 1995, I performed overnight-ligation of the restricted PCR fragments and vectors (RE93 , pages 46-47).

143. On July 11, 1995, I transformed the products of the ligations of July 10, 1995 into competent DH5α cells, and cultured the cells overnight, *i.e.*, until July 12, 1995 (I RE93 page 50).

144. On July 12, 1995, I picked colonies of transformed cells and placed the colonies individually into LB/Amp media for culturing (RE93 , page 52). Following a four-hour culture time, I performed PCR analysis on the cultured colonies to determine if the appropriate clones had been generated, and performed agarose gel electrophoresis of the PCR reaction products. None of the PCR reactions generated the appropriate product, so I repeated these

experiments over the time period of July 13, 1995 through July 17, 1995 (RE93 page 52).

145. On July 13, 1995, I did further PCR analysis of the colonies generated on July 12, 1995 and ran the PCR reaction products on a gel (RE93 , pages 54 and 59).

146. On July 17, 1995, I selected additional positive colonies and cultured them overnight in TB/Amp media (RE93 , page 64).

147. On July 18, 1995, I prepared minipreps of the July 17, 1994 cultures and set up overnight digests with BamHI/XhoI (RE93 , pages 64-65).

148. On July 19, 1995, I ran an agarose gel of the digests to isolate fragments (RE93 , page 68).

149. On July 20, 1995, I initiated midi preps of the HTPAN08/pcDNA constructs in order to scale up DNA production (RE93 page 72). I continued processing of these midi preps on July 21, 1995 (RE93 , page 75).

150. On July 24, 1995, I performed phenol: chloroform extraction and ethanol precipitation of the midi prep DNA in order to purify it. Samples of the three purified DNA midi preps were run on a gel. The DNA looked good, so I set up overnight digests using Bam HI and Xho I enzymes (RE93 , pages 76-77).

151. On July 25-26, 1995, the HGS Molecular Biology work areas were closed and I was consequently unable to perform work on the project on those dates (RE93 , pages 77-78).

152. On July 27, 1995, I ran samples of the DNA digests on a gel. I then submitted the DNA for sequencing with internal primers (RE93 , page 79).

153. On August 16, 1995, I performed transfection experiments to analyze the expression of the new constructs (RE93 , pages 108-09).

154. On August 17, 1995, I performed restriction digest and agarose gel analysis of the new constructs (RE93 , page 109).

Optimization of AIM-I Expression With Bacterial Constructs pQE60, pD10, pQE and pQE6 and Western Analyses

155. Under Dr. Ruben's supervision, I carried out a number of experiments designed to optimize bacterial expression and purification of the AIM-I protein. Example of such efforts up to March 14, 1996 are summarized below.

AIM-I Antibody Production

156. On March 8, 1995, I initiated a culture of HTPAN08/pD10 51bp for large-scale IPTG induction of the AIM-I protein (RE92 page 60).

157. On March 9, 1995, I induced expression of AIM-I protein in the culture initiated on March 8, 1995 (RE92 , page 62). Four hours following the induction, I spun down the cells and froze them overnight, *i.e.*, until March 10, 1995 (RE92 , page 62).

158. March 10, 1995, I performed PAGE analysis on the previous day's induction/expression experiment, and the results indicated that AIM-I protein (Met-1) was successfully produced in bacteria (RE92 , page 62). Also on March 10, 1995, I successfully purified AIM-I protein (Met-1) over an $NiSO_4$ column (RE92 , pages 62 and 69).

159. On March 14, 1995, I ran a preparative gel to purify additional AIM-I protein for antibody production (RE92 page 71). On March 15, 1995, I isolated AIM-I protein from a preparative gel slice. This AIM-I sample was produced from an HTPAN08S04 51bp construct by induction of bacterial expression on March 10, 1995 (RE92 , page 72, dated March 15, 1995).

160. On June 26, 1995, I prepared an additional sample of AIM-I protein, expressed from the HTPAN08SO4 51bp ATG+ construct, and sent it to Pocono Rabbit Farm and Laboratory (PRF&L) for anti-AIM-I antibody production (RE93 , page 28; RX 2024). In my letter dated June 26, 1995 to Cindy Haab of PRF&L, in which I referred to AIM-I as "FasLig", I requested that PRF&L produce anti-"FasLig" antibodies by its standard protocol for fusion protein antigens.

161. On June 27, 1995, I inoculated a small volume of media to start a large-scale induction of HTPAN08S04 51bp ATG +pD10 protein (RE93 , page 30).

162. On June 28, 1995, I expanded this culture by inoculation into of 600 ml of media. Also, I ran gels in preparation for a Western blot of induced, uninduced, and purified samples derived from HTPAN08S04 51bp ATG + pD10. The samples were transferred from the gel to nitrocellulose and stored (RE93 , pages 31-32).

163. On June 29, 1995, I ran the supernatant from the previous day's large scale preparation over a $NiSO_4$ column in order to purify the AIM-I protein by virtue of its N-terminal His tag (RE93 pages 34-35). This protocol was repeated on June 30, 1995 using freshly charged resin (RE93 , page 36).

164. I received the initial anti-AIM-I antisera from PRF&L rabbits 11940 and 11941 on August 30, 1995 (RE93 , page 129) which I tested in a Western blot, also on August 30, 1995 (RE93 , page 130). On August 30, 1995, I set up nonimmune sera from the pre-bleed and immune sera from test bleed #1 for overnight incubation with the Western blots generated on June 28, 1995 (RE93 , page 130). When I examined the blot on August 31, 1995, it showed a positive result in the induced protein lane using test bleed sera but not pre-bleed sera (RE93 page 131). This result indicated the binding of antibody to the AIM-I protein expressed from the HTPAN08S04 51bp ATG bacterial expression construct and confirmed the presence of antibodies specific for AIM-I in the initial, small antisera samples.

165. On August 31, 1995, I performed a PCR reaction to amplify HTPAN08 pQE60 bacterial expression constructs and ran the products on a gel (RE93 pages 132-133).

166. On September 1, 1995, the PCR products generated the previous day were again run on a gel RE93 page 133).

167. On August 31, 1995, I innoculated 50 ml of LB medium with another AIM-I expression construct (HTPAN08 51bpATG + pD10) for overnight incubation RE93 , page 133).

168. On September 1, 1995, I expanded the HTPAN08 51bpATG + pD10 culture into 300 ml LB/Amp media, induced AIM-I expression, and pelleted and resuspended the cells (RE93 , pages 133-34). The induction step caused the bacteria to express the AIM-I protein.

169. On September 5, 1995, I digested new AIM-I constructs (designated HTPAN08 51bpATG + pQE and HTPAN08 185bpATG + pQE) overnight with Nco/Bgl II restriction

enzymes (RE93 , page 137).

170. On September 6, 1995, I precipitated the digested DNA, and washed and ligated the DNA overnight with the pQE Nco/BgI digested vector (RE93 pages 137-38).

171. On September 7, 1995, I transformed the pQE ligations into M15 cells and plated for overnight incubation (RE93 , page 139).

172. On September 7, 1995, I also incubated AIM-I antisera overnight with a Western blot of AIM-I expressed from the HTPAN08 51bpATG construct (RE93 , page 139).

173. On September 8, 1995, I picked and cultured positive pQE colonies from the M15 cell transformation. I performed PCR on the colonies to confirm positives, which I transferred to culture (RE93 , pages 139-141).

174. On September 8, 1995, I also developed the Western blot from the previous day which showed positive signals in the induced lanes for antisera derived from both rabbits (RE93 pages 139-42). Additionally, the first large scale bleeds for antibodies 11940 and 11941 were received from PRF&L, and secondary antibodies were conjugated in preparation for Western blotting RE93 , pages 141).

175. On September 11, 1995, I induced the AIM-I/pQE bacterial expression cultures of September 8, 1995 and generated a protein gel, which I stained overnight (RE93 , pages 142-143).

176. On September 12, 1995, I destained the protein gel, revealing induction of AIM-I protein expression from the pQE 185bp ATG construct. I also performed boiling preps and digests and inoculated cultures with the pQE 51bp and 185bp constructs for overnight incubation

(RE93, pages 143-44).

177. On September 12, 1995, I performed PCR reactions to identity colonies positive for the pQE closes (RE93 , page 146).

178. On September 13, 1995, the previous day's PCR reactions I with different primers and run on a gel. Also, boiling minipreps of pQE were performed and run on a gel, and the DNA was set up for overnight digestion with NcoI/BglII (RE93 , pages 147 and 149).

179. On September 14, 1995, I ran the pQE NcoI/BglII digests on a gel (RE93 , page 152).

180. On September 14, 1995, I performed a Western blot on the pQE 51bp induction RE93 , pages 150, 152).

181. On September 15, 1995, I repeated the Western blots of pQE inductions (RE94 , page 3).

182. On October 4, 1995, I inoculated the M15 rep 5 strain of *E. coli* with the HTPAN08 pD10 construct, another bacterial expression vector, in order to conduct an experiment designed to determine the relative expression levels of four independent clones (designated D9, D7, C7 and A2) as part of an effort to optimize bacterial expression of AIM-1 (RE94 , page 32).

183. On October 6, 1995, I cultured clone D9 and induced the culture with IPTG. The resulting bacterial cells were pelleted and stored at 4°C (RE94 , page 40).

184. On October 9, 1995, I processed these bacterial cell pellets by column purification to obtain the induced AIM-1 protein (RE94 , page 42).

185. On October 10, 1995, I ran the protein obtained on a gel to analyze the elution characteristics of the column (RE94 , page 44).

186. On October 11-13, 1995, I repeated this procedure (RE94 , pages 45, 47-49).

187. On October 16, 1995, an AIM-I isolate was obtained and run on a gel ("pH 6 isolate"). This protein was excised from the gel and stored at 4°C (RE94 , page 50).

188. On October 25, 1995, more gels were run of purified AIM-I proteins expressed in bacteria. Samples of both the "Met-1 and "Met-45 AIM-I proteins were analyzed, including those purified above on October 11-13, 1995 (RE94 , page 61).

189. On October 26-27, 1995, I prepared to perform more Western blots using anti-AIM-I Abs 11940 and 11941 in order to quantitate the protein (RE94 , pages 62-69). On October 30-31, 1995, anti-AIM-I Ab 11940 dilutions were examined (RE94 , pages 68-69).

190. On November 1-2, 1995, primary antibody, secondary antibody, and substrate were added to develop the blots (RE94 , pages 70-72).

191. On December 4, 1995, subcloning of AIM-I into the pQE6 bacterial expression vector was begun. PCR primers were designed to incorporate Nco I and HindIII restriction sites into the HTPAN08S04 51bp (Met-I) open reading frame (ORF) for subcloning into the pQE6 bacterial expression vector. The construct was referred to as HTPAN08S04 51bp + PQE6. PCR was done to amplify the desired fragment, which was then digested with NcoI/HindIII for subcloning. Use of these primers eliminated the 6-His tag of the pQE vector (RE94 , pages 96-97).

192. On December 5-6, 1995, the digested inserts and vectors were isolated, ligated

and transformed into bacteria (RE94 pages 98-100). Colonies were screened by PCR for the presence of insert on December 7, 1995, and mini-preps were prepared to confirm the subcloning (RE94 , pages 100-05).

193. On December 11, 1995, small-scale IPTG-inductions in *E. coli* of the AIM-I sequence in the pQE6 expression vector was performed (RE94 , pages 106-07).

194. On December 12, 1995, the induced proteins were run on a gel. Clones were also analyzed by miniprep restriction analysis, and were submitted for sequencing to confirm the accuracy of the subcloning (RE94 , pages 108-09).

195. On December 15, 1995, the inserts from the AIM-I/pQE6 bacterial expression clones were PCR amplified to be used for *in vitro* protein expression in the TNT system (RE94 , page 116), which is detailed further, below.

196. On February 6, 1996, PCR primers were designed in order to subclone AIM-I into the pQE6 bacterial expression vector at a different ATG start codon, and bacteria were grown for a maxi-prep of HTPAN08/pQE6 construct DNA to accomplish this objective (RE95), pages 5, 7-11).

197. On February 8-9, 1996, PCR was performed to determine the optimal *Pfu* polymerase amplification conditions to amplify inserts for subcloning into a cloning vector, pBSK (primers 4632 and 14388) (RE95 , pages 8-14).

198. On February 12, 1996, restriction digests were performed in preparation for further subcloning (RE95 , page 20).

199. On February 13-16, 1996, AIM-I was again cloned into the pQE6 bacterial

expression vector at a BspHI/HindIII site. Maxi-preps of AIM-I DNA were also prepared (RE95 , pages 24-32).

200. On February 19-22, 1996, I transformed cells with the pQE6 construct carrying the AIM-I insert, grew them up, and analyzed the resultant DNA by PCR and gel electrophoresis to demonstrate the presence of the insert (RE95 , pages 33-38).

201. On February 27, 1996, Dr. Ruben's laboratory was packed and moved to a new location, precluding lab work by me on that date (RE95 , page 57).

202. On March 5-8, 1996, a third attempt was made to clone AIM-I into the pQE6 bacterial expression vector at a BspHI/HindIII site (RE95 , pages 71-72, 75, 76, 78-81).

Indirect Immunofluorescence With pcDNA Constructs

203. Under Dr. Ruben's supervision, I carried out indirect immunofluorescence analysis of the AIM-I protein as part of our overall effort to obtain a detailed understanding of the biological properties of AIM-I as they exist in a living mammalian cell. Generally, immunocytochemistry can be used to reveal the subcellular localization of a protein. Moreover, a protein under study can be genetically engineered to display antigen tags at various locations along the linear sequence of the protein, such as at the N-terminus and the C-terminus. The tagged protein can then be subjected to immunocytochemical analysis to reveal the subcellular localization of specific antigen-tagged portions of the protein. Such an approach can reveal important information about protein processing, topology, and orientation within cellular membranes. The immunofluorescence experiments set forth below were designed to determine the sub-cellular location of the AIM-I protein.

204. On September 25, 1995, I ethanol precipitated DNA encoding three mammalian AIM-I expression constructs in preparation for transfections. Specifically, the experiment employed: (a) pcDNA (a mammalian expression construct); (b) pcDNA3'HA (same as pcDNA, but with a hemagglutinin (HA) tag inserted in-frame at the C-terminus); and (c) pcDNA5'HA (same as pcDNA, but with an N-terminal HA tag) (I RE94 , page 20).

205. On September 26, 1995, I transfected COS cells with these three constructs (I RE94 , pages 21-22).

206. On September 28, 1995, the transfected cells were washed, fixed, blocked, and contacted with the primary anti-HA tag antibody (designated SCP-12CA5-J) for overnight incubation (RE94 pages 25-26).

207. On September 29, 1995, I removed the primary antibody, washed the transfected cells, and added the secondary antibody in order to visualize the cellular location of the AIM-I polypeptides expressed from the three transfected constructs (RE94 page 27).

208. On October 3, 1995, I repeated the immunofluorescence experiment to determine the sub-cellular location of AIM-I (I RE94 , pages 30-31). Cells were transfected on October 3, 1995 and incubated for 48 hours (*i.e.*, until October 5, 1995). On October 5, 1995, the transfected cells were washed, fixed, and incubated with primary antibody overnight (I RE94 pages 36-37). On October 6, 1995, the primary antibody was removed and the cells were treated with secondary antibody and stored (i RE94 , pages 38-39).

AIM-I Northerns

209. Northern blots, or RNA blots, provide an indication of the tissue specificity and cell specificity of expression of a gene such as AIM-I.

210. On December 7, 1995, I probed human RNA blots using a labeled HTPAN08S04 Xho/EcoRI fragment encoding AIM-I as probe. Two blots were probed: (a) a Multiple Tissue Northern (MTN) blot; and (b) a Cancer Tissue Northern blot (I RE94 pages 100-04).

211. On December 8, 1995, the results indicated only faintly detectable bands, so I reexposed the blots over the weekend (RE94 page 105).

212. On December 11, 1995, I again developed the blot exposures which revealed heavier bands (RE94 , pages 106-107).

Patent Related Activity

213. On February 12, 1996, I aliquoted 25 samples of 100 µg of HTPAN08SO4 plasmid DNA and gave them to Dr. Ruben for him to deliver to the HGS Legal Department to make a deposit in connection with the filing of an AIM-I patent application (RE95 , page 20).

Holidays, Weather-Related Closings, and Vacation Days

214. During the 1995 and 1996 time frame, HGS observed the practice of being closed on Federal Holidays. The closure on Federal Holidays that occurred during critical period were noted by me in my notebooks. In addition, HGS was closed according to its usual practice for the entire week between the Christmas and New Year's Day holidays. Moreover, the blizzard of

January 1996 caused HGS (like the Federal Government) to close for four out of five days during the week of January 8-12, 1996. In addition, I was primarily responsible for the hands-on work in cloning and characterizing AIM-I. took reasonable vacation time during the year, and spent one day packing and moving the laboratory, as noted in the chronology below. On the following days, I could not carry out work for the reasons indicated:

May 26, 1995, Ann Kim Day Off (RE92 page 141);

July 4, 1995: Independence Day Holiday (RE93 page 40);

On July 25-26, 1995, the HGS Molecular Biology work areas were closed due to a radiation problem (RE93 . pages 77-78).

September 4, 1995: Labor Day (RE93 , page 134);

November 22, 1995: I was out of the office (RE94 , page 90);

November 23-24, 1995: Thanksgiving Holiday (RE94 , page 90)

December 22, 1995: I was on vacation (RE94 , page 127);

December 25, 1995- January 1, 1996: HGS closed for Christmas/New Year's (RE94 , page 127);

January 2-5, 1996: I was on vacation (RE94 , page 127);

January 8-10 and 12, 1996: Blizzard - HGS closed (RE94 pages 127, 128);

January 29 - February 2, 1996: I was on vacation (RE94 , page 152);

February 19, 1996: Presidents Day Holiday (RE95 , page 33);

February 28, 1996: Dr. Ruben's laboratory packed and moved, preventing research (RE95 , page 57).

215. I hereby declare that all statements made herein of my own knowledge are true

and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application captioned above or any patent issuing thereupon.

June 24, 2004
Date:

Ann Ferrie